ACTION OF VARIOUS ENZYMES ON THE MATING TYPE SUBSTANCES OF PARAMECIUM CALKINSI[1]

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When paramecia of complementary mating type are mixed under appropriate conditions, these animals adhere to one another and shortly form large clumps or agglutinates. All available evidence (Kimball, 1943; Metz, 1948) indicates that this initial agglutinative mating reaction results from interaction of complementary substances or at least complementary configurations on the surfaces of the cilia. For convenience these substances or configurations are called the mating type substances.

Recent studies have shown that interaction of mating type substances may perform a far more significant function in fertilization than the obvious one of effecting the initial union of potential conjugants. Thus, Metz and Foley (1949) using a mutant stock of *Paramecium aurelia* found that only the initial mating reaction union is essential for induction of meiosis, macronuclear breakdown, loss of mating reactivity and pseudo selfing pair formation in this species. Since the mating reaction alone was essential for induction of these activation phenomena, it seemed reasonable to conclude that interaction of mating type substances initiates the activation. In view of this, it is of interest to obtain more specific information regarding the physical basis for the mating reaction, in other words to define the mating type substances in more concrete physico-chemical terms. In an attempt to achieve this goal an investigation of the chemical nature of the mating type substances has been undertaken. As one phase of this study several enzymes were tested for action on the mating activity of paramecium. *Paramecium calkinsi* was selected for this study because of the ease with which cultures of this species may be obtained in mating condition. Of the several enzymes tested, only the proteolytic enzymes, trypsin and chymotrypsin, affected the mating reactivity of the paramecia.

Material and Methods

Enzyme preparations. The following enzyme preparations were tested for their effects on the mating reactivity of *P. calkinsi*: crystalline preparations (Armour) of trypsin, chymotrypsin, lysozyme and ribonuclease; purified hyaluronidase and crude preparations of ptyalin and lecithinase (bee venom, prepared by the method of Flury, 1920).

Preparation of the paramecia for testing. As pointed out elsewhere (Metz and Butterfield, 1950), paramecia in mating condition quickly lose their mating reactivity when fed bacteria. Furthermore, paramecia appear to feed upon concentrated protein solutions (enzymes, rabbit sera) since they enlarge, become filled with food vacuoles and sometimes divide in such solutions. To avoid the possibility of such

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indirect loss of mating activity from feeding, the paramecia were killed with formalin or picric acid before treatment with enzyme. Paramecia killed by appropriate treatment with these agents give strong, specific mating reactions. Indeed, such dead animals can activate living animals of opposite type (Metz, 1948). The reader is referred to the article of Metz and Butterfield (1950) for the methods of culturing and killing the *P. calkinsi*.

Method of treatment with enzyme. To test for action on the mating substance, both the enzyme preparation and the formalin- or picric acid-killed reactive animals were taken up in an appropriate buffer with pH adjusted near the optimum for enzyme action. Equal volumes of enzyme solution and paramecium suspension were then mixed in depression slides or precipitin tubes. After an appropriate incubation period (1–6 hrs., 20°–30° C.) the treated animals were washed free of enzyme solution with $\frac{2}{3}$ sea water and tested for mating reactivity with living reactive type I and II animals. To control for reactivity of the dead animals and to check for inactivation of the animals by the buffer, equivalent samples of the same dead paramecia were treated with buffer but without enzyme solution. When appropriate known enzyme substrates were available, these were employed as controls for enzyme activity. To accomplish this, samples of the enzyme solution and a solution of appropriate known substrate in the same buffer were mixed and incubated in parallel with the treated paramecia. In a few cases the experiment was set up in such a manner that the enzyme digested an amount of known substrate equal to or greater than the mass of the enzyme treated paramecia. The mass of the paramecia was determined roughly by counting the animals present and multiplying by 0.05, the weight (in micrograms) of one *Paramecium calkinsi*. This figure was obtained from the number (7.5×10^5) of paramecia in a 37.3 mg. sample of washed, lyophylized animals. It agrees reasonably well with the figures of Boell and Woodruff (1941).

As a final control, the supernatant buffer from a sample of the dead paramecia usually was tested for inhibition of enzyme action. Conceivably substances leaching from the dead animals could inhibit the enzyme in the test sample. Traces of the formalin or picric acid used to kill the paramecia would fall into this category. In all cases where this control was employed, the enzyme was active in the animal supernatants.

Results

Non-proteolytic enzymes. Crystalline (Armour) preparations of lysozyme and ribonuclease, purified (1300 turbidity reducing units/mg.) hyaluronidase and crude preparations of ptyalin and lecithinase (bee venom) all had no detectable effect upon the mating reactivity of dead *P. calkinsi*. The conditions under which each of these enzymes was tested are given in Table I.

Proteolytic enzymes. In marked contrast to the preparations considered above, the proteolytic enzymes trypsin and chymotrypsin destroyed the mating reactivity of formalin- or picric acid-killed *Paramecium calkinsi* of both mating types. Five-tenths per cent crystalline (Armour) trypsin and chymotrypsin solutions were made up in borate buffer, pH 8. Equal volumes of these enzyme solutions and suspensions of dead paramecia in the same buffer were mixed and allowed to stand. At intervals samples of dead animals were removed from the enzyme solution, washed in $\frac{2}{3}$ sea water and tested for mating reactivity with living type I and II test

animals. A portion of each sample was also stained with iodine solution and examined under the oil immersion objective for the condition of the cilia. Dead animals from the same original sample but treated with buffer only (no enzyme) served as the standard of comparison both for mating reactivity and condition of the cilia. The results of a typical experiment with trypsin and chymotrypsin are given in Table II. It is evident from the data of Table II that the trypsin and chymotrypsin preparations destroy the mating reactivity of both type I and II animals.

The striking difference in time for inactivation of formalin-killed as opposed to picric acid-killed animals is not readily explained. Possibly this difference in in-

TABLE I

Conditions under which non-proteolytic enzymes were tested for action on formalin- and picric acid-killed Paramecium calkinsi

Enzyme	Animals Treated*	Buffer	pH	Final Enzyme Concentration	Temperature	Digestion Time	Inhibition of Enzyme by Supernate	Mg. Animals Treated with Enzyme	Mg. Known Substrate Digested
Bee venom	F I	2.5 sea water	6.9	0.63%	28° C.	1.5 hrs.	No inhibition	0.045	Hemolyzed 2% RBC suspension
Hyaluronidase[2]	F I	2.5 sea water	6.9	0.63%	28° C.	2 hrs.		0.018	
Lysozyme	F I	0.0025 N NaCl	6.6	0.5%	Room temp.	2.7 hrs.	No inhibition	>1.0	Lysis of turbid *S. lutea* suspension
	P I	NaCl	6.6	0.5%	Room temp.	2.6 hrs.		>1.0	
	P II	NaCl	6.6	0.5%	25° C.	2.0 hrs.		>1.0	
Ptyalin	F I, P II	Phosphate	6.6	1/2 sat. sol.	37° C.	2.0 hrs.	No inhibition	0.4	0.6 mg. soluble starch
Ribonuclease[3]	F I	Borate	8.0	0.075%	30° C.	6 hrs.	No inhibition	0.1 mg. paramecium nitrogen	0.7 mg. Na nucleate nitrogen >5 mg. Na nucleate
	P I, P II	Borate	8.0	0.05%	Room temp.	6 hrs.			

* F I indicates formalin-killed type I animals; P I, picric acid-killed type I animals; and P II, picric acid-killed type II paramecia.

[2] The authors are indebted to Dr. J. Seifter, Wyeth Institute of Applied Biochemistry, for this preparation of hyaluronidase.

[3] The ribonuclease preparation was found to hydrolyze casein. This proteolytic action was removed by the method of McDonald (1948).

activation time simply reflects a difference in the number of reactive groups exposed on the surfaces of the paramecia. This view is supported by the fact that formalin-killed animals usually give stronger mating reactions than picric acid-killed animals. Possibly traces of formalin leaching from the dead animals inhibit the enzymes to a greater degree than corresponding traces of picric acid. Finally, differences in the extent of alteration ("denaturation") of paramecium proteins by formalin, as opposed to picric acid, may account for these results. In this connection it will be recalled that denatured proteins are known to be more susceptible to the action of proteolytic enzymes than the native material (Haurowitz, 1950).

As might be expected, the enzyme preparations not only destroy the mating reactivity of the dead animals but in time they digest the cilia and eventually reduce

the formalin- or picric acid-killed animals to fragile ghosts. However, the enzyme-treated dead animals can lose their ability to give the mating reaction before the cilia show any appreciable action of the enzyme. Cilia of control formalin- or picric acid-killed paramecia are usually straight and extend in a radial direction from the body of the animal. After treatment with trypsin or chymotrypsin, however, the cilia (iodine stained) gradually lose sharpness of outline, become curly and finally disappear. The picric acid-killed animals of Table II still possessed cilia after a 17 minute treatment with trypsin. However, the cilia of these animals could be distinguished from those of the controls. The former were classified as "fair to good" (picric acid-killed type I animals at 17 minutes) or "poor to fair" (picric acid-killed type II animals) when compared for sharpness of outline and straightness with the controls. Likewise, picric acid-killed, chymotrypsin-treated animals retained their cilia for some time following loss of mating activity. Even at 70 minutes these type I animals (Table II) possessed fair to good cilia. The cilia of some animals were indistinguishable from those of controls. At 165 minutes,

TABLE II

Mating reactivity of trypsin- and chymotrypsin-treated dead Paramecium calkinsi

Enzyme	Digestion Time	Enzyme Treated			Buffer Treated		
		Formalin-killed I	Picric Acid-killed		Formalin-killed I	Picric Acid-killed	
			I	II		I	II
Trypsin	17 min.	++++	—	—	++++	++-+++	+++
	62 min.	++	—	—	++++	++	++
	137 min.	++	—	—	++++	++	++-+++
Chymo-trypsin	25 min.	+++	—	+	++++	++	+++
	70 min.	++	—	—	++++	++	+++
	165 min.	++	—	—	++++	++	++

however, these animals were devoid of cilia. The picric acid-killed type II animals had "poor to fair" cilia after 60 minutes treatment with chymotrypsin and no cilia after 165 minutes treatment.

From these and other observations on trypsin and chymotrypsin treated animals, it is evident that loss of mating activity does not necessarily involve any serious visible change in the structure of the cilium. Thus, the loss of mating reactivity effected by these enzymes results initially from an alteration in the surface structure of these organelles. Interpretation of this effect in terms of a specific protein substrate presents certain difficulties, however. The proteolytic enzymes could act directly on the reactive groups (*i.e.*, the "mating substance"), in which case these reactive groups would be proteinaceous. On the other hand, the enzymes might destroy mating reactivity indirectly by breaking down protein in the superficial layers of the cilium. Such action might alter the relations (*i.e.*, spacing) of essential non-protein surface groups or even release the essential material into solution. This latter possibility was tested as outlined in the following section.

Failure of trypsin digests of reactive paramecia to act on animals of opposite type. Reactive *P. calkinsi* type I in four liters of culture were treated with formalin. The resulting reactive dead animals were washed in saline and pH 8 phosphate buffer. The final yield of 0.7 ml. of packed dead animals was taken up in one ml. of the buffer. Five-tenths ml. of this sample was mixed with an equal volume of 0.5 per cent trypsin in the same buffer. The remaining 0.5 ml. of suspension (control) was treated with buffer alone. The two suspensions were incubated at 32° C. for 5 hours. At the termination of this digestion period the enzyme-treated animals were reduced to fragile ghosts which gave no mating reactions. Animals of the control sample (treated with buffer alone) appeared to be in a good state of preservation and gave good mating reactions. The unreactive ghosts were now centrifuged from the trypsin-treated suspension and 0.25 ml. of one per cent trypsin inhibitor (ovomucoid prepared as described by Lineweaver and Murray, 1947) was added to prevent action of the enzyme on test animals. This trypsin inhibitor-treated, trypsin digest of formalin-killed *P. calkinsi* type I was tested for its ability selectively to destroy mating reactivity of type II animals. Eight drop samples of the digest were mixed with three drop samples from suspensions of diluted, freshly prepared, picric acid-killed reactive type I and II animals. One hour later these dead animals were washed in saline and tested for mating reactivity. The digest-treated type I and II animals both gave mating reactions of intensity equal to that of buffer-treated controls. Evidently, then, the trypsin digest of reactive type I animals has no mating reaction inhibiting properties.

Discussion

None of the several non-proteolytic enzymes employed had any detectable effect upon the mating reactivity of the paramecia. Assuming that these experiments were critical, this result indicates that the natural substrates of these enzymes are not essential constituents of the mating type substances of *P. calkinsi.* Elimination of these classes of substances as possibilities is not without interest since certain of them are known to be present in intercellular cement (hyaluronic acid) or cell membranes and cell surfaces (bacterial mucopolysaccharides hydrolyzed by lysozyme, Meyer and Hahnel, 1946; ribonucleic acid in Arbacia egg surface, Lansing and Rosenthal, 1949; lipoprotein attacked by lecithinase in erythrocyte and sea urchin egg membranes, Runnström, Tiselius and Lindvall, 1945).

As suggested elsewhere in this article the inactivating action of trypsin and chymotrypsin can be interpreted either as a direct effect upon the essential groups of the mating substance or an indirect action upon neighboring protein. Since no mating substance activity could be demonstrated either from the animals or the supernatant fluid following treatment with these enzymes, it appears that some essential molecular structure was destroyed. This suggests that the essential groups are protein or that they are intimately associated with protein. Insofar as the presence of immunizing antigens indicates protein, the immunological studies of Metz and Fusco (1948) support this view. It is of interest in this connection that the latter study indicates that the mating substance is associated with antigenic material but that the essential groups are not themselves antigenic.

Unfortunately, no more positive statement regarding the chemical nature of the mating substance seems warranted on the basis of the information presented here.

If mating substance activity is dependent upon protein integrity, as seems to be the case, then the protein material must be of rather unusual character. Its activity is not destroyed by fairly harsh treatment with certain strong protein-denaturing agents such as formalin, picric acid and mercuric chloride. Furthermore, the rate of inactivation of mating substance by trypsin and chymotrypsin may depend upon previous treatment (*i.e.*, formalin vs. picric acid). If true, this would also be an unusual situation since any substance or group of substances with as complex and highly specific action as these would be expected to show some differences in activity to correspond with differences in susceptibility to enzymes. Unfortunately no satisfactory method has been devised to assay mating substance activity. Therefore serious quantitative studies of the reactivity of formalin- as opposed to picric acid-killed animals, both before and after enzyme treatment, must await development of an appropriate technique. In view of these considerations, the possibility that the mating type substances of *P. calkinsi* are proteins is accepted as a working hypothesis with the customary reservations.

Aside from the question of the chemical nature of the mating type substances, the mating substance inactivating action of proteolytic enzymes is of interest in connection with the inhibiting action of certain paramecium extracts. Metz and Butterfield (1950) have shown that homogenates of *P. calkinsi* contain a heat-labile, non-type specific mating substance inhibiting agent. It was suggested that this agent might be an enzyme. Considering the present findings it seems possible that the agent in question is a proteolytic enzyme. Such an enzyme need not bear any physiological relation to the mating substances. Indeed, it may well be a proteolytic enzyme which is released into the food vacuoles in the living animals and which under normal conditions participates in the digestion of the contents of these vacuoles (see Mast, 1947). Upon homogenizing the paramecia, such an enzyme could be released into the extraction fluid and under the conditions of the experiments would be expected to inactivate the mating substances of the test animals.

Summary

1. In an attempt to characterize the mating type substances of *Paramecium calkinsi*, several enzymes were tested for ability to destroy the reactivity of formalin- or picric acid-killed animals.

2. The following non-proteolytic enzymes had no detectable effect upon the mating reactivity of *P. calkinsi*: Lecithinase (bee venom), hyaluronidase, lysozyme, ptyalin, ribonuclease. Insofar as these experiments were critical, it is concluded that the natural substrates for these enzymes are not essential constituents of the mating type substance of *P. calkinsi*.

3. Crystalline preparations of the proteolytic enzymes trypsin and chymotrypsin destroyed the mating reactivity of both type I and type II animals. Mating substance activity was not found in the digest of enzyme-treated paramecium.

4. On the basis of these findings it is concluded that mating reactivity is dependent upon protein integrity. Possibilities regarding the nature of this dependence are discussed.

LITERATURE CITED

BOELL, E. J., AND L. L. WOODRUFF, 1941. Respiratory metabolism of mating types in *Paramecium calkinsi*. *J. Exp. Zool.*, **87**: 385–402.

FLURY, F., 1920. Über die chemische Natur des Beinengiftes. *Arch. Exp. Path. Pharm.*, **85**: 319–338.

HAUROWITZ, F., 1950. Chemistry and biology of proteins. Academic Press, New York.

KIMBALL, R. F., 1943. Mating types in the ciliate protozoa. *Quart. Rev. Biol.*, **18**: 30–45.

LINEWEAVER, H., AND C. W. MURRAY, 1947. Identification of the trypsin inhibitor of egg white with ovomucoid. *J. Biol. Chem.*, **171**: 565–581.

MCDONALD, M. R., 1948. A method for the preparation of "protease free" crystalline ribonuclease. *J. Gen. Physiol.*, **32**: 39–42.

MAST, S. O., 1947. The food vacuole in Paramecium. *Biol. Bull.*, **92**: 31–72.

METZ, C. B., 1948. The nature and mode of action of the mating type substances. *Amer. Nat.*, **82**: 85–95.

METZ, C. B., AND W. BUTTERFIELD, 1950. Extraction of a mating reaction inhibiting agent from *Paramecium calkinsi*. *Proc. Nat. Acad. Sci.*, **36**: 268–271.

METZ, C. B., AND M. T. FOLEY, 1949. Fertilization studies on *Paramecium aurelia*: an experimental analysis of a non-conjugating stock. *J. Exp. Zool.*, **112**: 505–528.

METZ, C. B., AND E. M. FUSCO, 1948. Inhibition of the mating reaction in Paramecium with antiserum. *Anat. Rec.*, **101**: 654–655.

MEYER, K., AND E. HAHNEL, 1946. The estimation of lysozyme by a viscosimetric method. *J. Biol. Chem.*, **163**: 723–732.

LANSING, A. I., AND T. B. ROSENTHAL, 1949. Ribonucleic acid at cell surfaces and its possible significance. *Biol. Bull.*, **97**: 263.

RUNNSTRÖM, J., A. TISELIUS AND S. LINDVALL, 1945. The action of androgamone III on the sea urchin egg. *Ark. Zool.*, 36A, No. 22: 1–25.